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DISCOVERY

# Evaluating Methods of Rat Euthanasia on the Liver and Kidney of Wistar rats: Cervical dislocation, chloroform inhalation, diethyl ether inhalation and formalin inhalation

**Aguwa Ugochukwu Samuel**[1]✉, **Okeke Somadina Nnamdi**[1], **Ezejindu Darmian Nnabuihe**[1], **Eze Chinyere Elizabeth**[1], **Okoro Janeth**[1], **Obinwa Benedict Nzube**[1], **Ovie Felix O**[2], **Obi Kelvin Chukwuemeka**[3], **Ogbuokiri Doris Kasarachi**[1], **Okeke Chijioke**[1], **Izuogu Morris**[1]

1. Anatomy Department, Faculty of Basic Medical Sciences, Nnamdi Azikiwe University, Nnewi
2. Anatomy Department, Faculty of Basic Medical Sciences, Abia State University, Uturu
3. Anatomy Department, Faculty of Basic Medical Sciences, University of Abuja

✉**Corresponding Author:**
Aguwa Ugochukwu Samuel. Email: usaguwa@gmail.com; +234-7030885803

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## ABSTRACT

A lot of procedural errors are committed at the point of sacrifice of research animals. This study compared four common methods of rodent sacrifice and their effects on the liver and kidney of Wistar rats. A questionnaire was distributed among Experimental and Clinical Anatomists of Nigeria (SECAN) during their annual conference as part of the preliminary studies. Twenty male Wistar rats weighing 160 to 200g were separated into four groups of five rats each. After two weeks of acclimatization, the animals were sacrificed; Group A by Cervical dislocation (control), Group B chloroform inhalation, Group C diethyl ether inhalation and group D Formalin inhalation. Blood was collected by cardiac puncture for serum chemistry. The liver and kidneys were harvested and fixed in 10% formalin for histological studies. Result of our preliminary studies showed that 90% of our scientists use rats for their studies, out of which 50% sacrifice their animals using chloroform sedation. Fifteen percent (15%) use formalin inhalation while 6.25% and 16.255 use diethyl ether and cervical dislocation respectively. Our results show that the methods of sacrifice showed adverse effect on the liver function test and kidney function tests of the rats except chloroform sedation that significantly increased alanine aminotransferase levels. However, Histological studies showed that while cervical dislocation showed no adverse effects, chloroform, diethyl ether and formalin inhalation showed various forms of cyto-architectural distortions on the liver and kidney tissues and should therefore be discouraged or used with caution.


**Keywords**: Cervical Dislocation, Chloroform, Dithyl ether, Formalin, Sacrifice.

## 1. INTRODUCTION

Animal sacrifice is a method of inducing humane death in an animal by a method that results in rapid loss of consciousness and death for research purposes (Fu et al., 2013). Sacrificing research animals is one of the most challenging tasks in animal studies, and it is imperative to do it as humanely as possible (Cressey, 2013). In the course of research, the fate of almost all experimental animals is to be sacrificed at certain stages of each study, either to gain blood, tissue, and other specimens (Marquardt *et al*., 2018).

Sacrificing techniques ultimately cause death by known mechanisms, including direct depression of neuronal activity necessary for life function, hypoxia and/or physical disruption of brain activity (Nolan *et al*., 2012). Common methods of animal sacrifice include; inhalation of anesthesia gas like chloroform, or carbon (IV) oxide, immersion agents, decapitation, injectable barbiturate agents, exsanguinations (AVMA, 2013).

Different researchers are of the opinion that particular methods of animal sacrifice like chloroform sedation is more ideal for certain research protocol and not for others. Inhaled anesthetics, beginning with diethyl ether, were first introduced into clinical practice in the 1840s. Since then a wide variety of inhaled agents, including ethers, alkanes, nitrous oxide, cyclopropane, and xenon, have been used to induce unconsciousness, amnesia, and immobility (Andrew *et al*., 2019).

Chloroform (trichloromethane) is a sweet-smelling volatile anesthetic that can be used for inhalational induction. Although it was initially developed as an alternative to ether, chloroform was abandoned because of its association with hepatotoxicity and fatal cardiac arrhythmias (Andrew *et al*., 2019). Chloroform sedation has been used to sacrifice animals with apparently no undesirable effects. However, reports have shown that chloroform has significant toxicity, including carcinogenicity, hepato-, and nephrotoxicity (Franks, 2006).

Carbon (IV) oxide, one of the most commonly used chemicals to sacrifice rodents, has been shown to cause behavioral aversion in all species tested. The aversion is speculated to be due to increased anxiety associated with the inability to escape (Shusterman *et al*., 2003). Carbon (IV) oxide-induced acidosis also evokes fear responses in mice (Ziemann *et al.,* 2009). It can also cause dyspnoea in rodents (Hawkins *et al*., 2006).

Physical techniques, such as cervical dislocation, which have been assumed to be humane methods for the sacrifice of small rodents, have been shown to have a surprisingly high failure rate (Carbone *et al*., 2012). Most physical methods have some limited evidence that they may not immediately abolish brain activity or consciousness (National Institutes of Health, 1986).

Barbiturates are used extensively and are considered the agents of choice for most animal research but barbiturates reduce cerebral metabolism leading to decreases in cerebral blood flow and intracranial pressure. They also produce respiratory depression and can elicit dose-dependent decreases in blood pressure (Franks, 2006).

The argument persists among researchers as to the most humane method of sacrificing experimental animals (Cressey, 2013). This study is therefore aimed at determining the most humane method of rat sacrifice as well as the method of sacrifice safest for liver and kidney studies. Alanine transaminase (ALT) is an enzyme that helps metabolize protein. When the liver is damaged, ALT is increased in liver and released in the bloodstream. Aspartate transaminase (AST) is an enzyme that plays a role in the metabolism of

the amino acid alanine. An increase in AST levels may indicate liver damage or disease. AST is a mitochondrial enzyme, and present in the liver, skeletal muscles and kidneys. Alanine transaminase (ALT) is a cytosolic enzyme, which is more specific for the liver than AST and alkaline phosphatase (ALP) (Hodgson, 2004; Aguwa et al., 2016.). ALT and AST in liver cells may leak out into the general circulation when liver cells are injured. ALT and AST are present in highest concentrations in cells from the liver, heart, skeletal muscles, and red blood cells (Infante, 2008). ALT is found predominately in the liver, with lesser quantities found in the kidneys, heart, and skeletal muscle. As a result, the ALT is a more specific indicator of liver inflammation than the AST, as the AST may also be elevated in diseases affecting other organs, such as the heart or muscles.

Healthy kidneys remove wastes and excess fluid from the blood. Blood and urine tests show how well the kidneys are doing their job. Creatinine is a waste product that comes from the normal wear and tear on muscles of the body. Creatinine levels in the blood can vary depending on age, race and body size. A high creatinine level may be an early sign that the kidneys are not working properly. The level of creatinine in the blood rises, if kidney disease progresses. Urea nitrogen on the other hand comes from the breakdown of protein. As kidney function decreases, the blood urea nitrogen level rises.

## 2. MATERIALS AND METHODS

### Materials

This research was carried out in the Department of Anatomy, College of Health Sciences, Nnamdi Azikiwe University, Nnewi Campus. Materials used include; dissecting kit, Electronic weighing balance (Leica CT 250 1101735428, 2012), Absorbent cotton wool, 10% formal saline, Haematoxylin and eosin (H and E), Dissecting pins, Dash board, 20 adult Wistar rats, wooden cages, Drinkers (plastic), Pyrex glass Beaker (100ml, 250ml and 500ml capacity), Measuring cynlinder (100ml capacity), Distilled water, Plates (for feeding) and Saw dust/wood sharing, light microscope, spectrophotometer.

### Preliminary Studies

A preliminary research was carried out to ascertain the commonest animals used for research among clinical anatomists as well as the prevalent method of sacrifice used by them. A questionnaire was distributed at the 18$^{th}$ annual conference and AGM of the Society of Experimental and Clinical Anatomists of Nigeria (SECAN) held at Enugu State University of Science and Technology (ESUT) held between the 28$^{th}$ to 30$^{th}$ of November, 2019. Participants for this conference were drawn from across Nigerian universities. A total of 141 questionnaires were distributed randomly among the participants out of which 126 were retrieved, representing 89.4%. Information obtained was relevant in making important research decisions.

### Animal subjects, grouping and treatment:

Twenty adult male Wistar rats weighing between 150 to 180g were used for this study. The rats were housed in standard wooden cages with wire gauze all around and chipping saw dust as bedding. The rats were fed pelletized rat feed produced by Pfizer. Animals were separated into 4 groups of five (5) rats each based on closeness to weight. The rats were acclimatized for 2 weeks under standard conditions for handling research animals. After acclimatization, the rats were sacrificed as follows: Group A by cervical dislocation, Group B by chloroform inhalation, Group C by Diethyl ether inhalation and Group D by formalin Inhalation.

For the cervical dislocation, the animals were suspended on their neck and pulled on the tail until the crack sound indicating dislocation of the atlantoaxial joint was confirmed. For the inhalation methods, the animals were introduced into a desiccator containing their corresponding chemical. Prior to exposure, the chemical was soaked in cotton wool and placed in the desiccator for 5minutes to ensure circulation. All chemicals used were manufactured by SIGMA-ADRICH, INC., P.O. Box 14508, St. Louis, MO 63178, USA and obtained from the biochemistry lab of Nnamdi Azikiwe University, Nnewi campus.

### Data / Sample collection and analysis:

Blood samples were collected from 5 rats in each group into ethylene diamine tetra acetic (EDTA) acid treated sample bottles for the determination of serum Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), and Alkaline phosphatase (ALP) for the liver as well as urea (UR) and creatinine (CR) for the kidney using standard procedures (Reitman & Frankel, 1957). Blood for hematological parameters were obtained from the retro-ocular plexus using heparinized capillary tubes prior to sacrifice. The serum chemistry was carried out at the Veterinary Pathology Laboratory of Michael Okpara University of Agriculture Umudike. These five rats also had their livers and kidneys harvested and fixed for histological analysis with H&E. The variation in the different groups was compared using the control as reference. The variations between the different groups for liver enzymes were compared using the student T-test. Liver and kidney function tests were determined using Randox kit following the manufacturer's instruction according to the method of Reitman & Frankel, (1957).

**Determination of serum ALT, AST and ALP levels**

Alanine aminotransferase is measured by monitoring the concentration of pyruvate hydrazine formed with 2,4-diphenylhydrazine according to the reaction below:

$$\alpha\text{-oxoglutarate} + \text{L- alanine} \xrightarrow{\text{GPT}} \text{L- glutarate} + \text{Pyruvate}$$

AST was assayed using Randox AST kit following the manufacturer's instruction according to the method of Reitman & Frankel, (1957). AST is measured by monitoring the concentration of oxaloacetate hydrazine formed with 2,4-dinitrophenylhydrazine as shown in the equation below;

$$\alpha\text{- oxoglutarate} + \text{L- aspartate} \xrightarrow{\text{GOT}} \text{L- glutamate} + \text{Oxaloacetate}$$

ALP was assayed using Randox AST kit following the manufacturer's instruction according to the method of Reitman & Frankel (1957).

$$\text{P-nitrophenylphosphate} + H_2O \xrightarrow{\text{ALP}} \text{Phosphate} + \text{P-nitrophenol}$$

**Determination of serumcreatinine and urea levels**

Serum creatinine level was determined using Human$^R$ reagent kits according to the method of Jaffe. Absorbance was measured at 546 nm by photometer (Techno 168). Serum urea on the other hand was determined by spectrophotometer using urease enzyme kit. Absorbance was measured at 578 nm.

**Histological analysis**

Tissues were fixed in 10% formal saline after sacrifice. Liver and kidney tissues were processed and embedded in molten paraffin wax. Five microns thick sections were made and stained with haematoxylin and eosin (H&E).Stained tissues were then examined under the research light microscope and micrographs taken.

**Statistical analysis**

Data obtained from serum chemistry were analyzed using one-way ANOVA using SPSS (version 21.0). Values were considered statistically significant at $P \leq 0.05$.

## 3. RESULTS AND DISCUSSION

**Result of preliminary findings**

The result of our preliminary studies proves that 90% of experimental anatomists use rats for their animal studies. Only about 2.5% use mice and the remaining 7.5% use rabbits. This informed our choice of using rats for this study because we seek a truly representative outcome. We also found that about 16.25% of our respondents sacrifice their animals by cervical dislocation, 50% by chloroform inhalation, 6.25% use diethyl ether inhalation while 15% use formalin inhalation.

Over 55% of our respondents have been actively involved in research for upwards of six (6) years and have used these methods over time. Majority of the respondents also agreed that their choice of method of sacrifice was based on availability of chemicals and equipment in the laboratory where the animal sacrifice took place. There is therefore the possibility that the researcher may be aware of the inappropriateness of the method, but has no better alternatives. Our findings also show that this error cuts across researchers with different years of experiences and rankings. It is a fundamental problem in Africa owing to poor funding for research and poorly equipped laboratories.

**Results of Liver function tests (LFTs)**

Our result in table i shows no statistically significant difference in the serum ALP and ALT levels in the Wistar rats. Also, AST levels were similar to those of the control except in group B where it was significantly increased compared to the control group.

Based on these reports, none of the methods of sacrifice interfered with the liver function enzymes of Wistar rats. However, the significant elevation in AST levels in the chloroform group may be indicative of an underlying destructive process happening not just in the liver, but on other organs in the rat. AST may also be elevated in diseases affecting other organs, such as the heart or muscles

(Hodgson, 2004). Aguwa (2016) reported that ALT is a better indicator of liver damage than ALP and AST. This result could imply that all the methods of sacrifice were relatively safe for liver studies, but chloroform inhalation was shown to pose more threat to the liver than the other methods. This is in line with the report of Goodies et al. (2015) which reported that chloroform and diethyl ether inhalation used in rat sacrifice had no effect on fasted blood glucose level and haematological parameters in Wistar rats. Also, the works of Plate et al. (2005) reports that diethyl ether when used for a short time as is the case in experimental animal sacrifice did not affect P450 enzymes activity.

**Table i**: Results of Liver Function Tests

| GROUP | AST (U/L) | ALP (U/L) | ALT (U/L) |
|---|---|---|---|
| **A** | 61.20±3.56 | 43.40±5.59 | 28.40±3.21 |
| **B** | 70.40±6.73* | 30.60±4.04 | 28.60±3.58 |
| **C** | 61.40±6.84 | 30.20±3.27 | 23.00±1.58 |
| **D** | 53.40±10.26 | 32.80±2.77 | 22.00±4.47 |

Values are expressed as Mean ± SD (n=5) *Indicate statistical significance at P≤0.05.

AST – Aspartate aminotransferase, ALP – Alkaline phosphatase, ALT – Alanine aminotransferase

U/L – units per liter

**Results of Kidney function test (KFTs)**

**Table ii**: Results of the Kidney Function Test

| Group | A | B | C | D |
|---|---|---|---|---|
| **Urea (mg /dl)** | 4.94±1.73 | 5.70±1.46 | 5.74±1.46 | 6.80±1.52 |
| **Creatinine (mg /dl)** | 146.60±28.41 | 153.40±22.63 | 153.00±18.36 | 164.80±10.99 |

Values are expressed as Mean ± SD (n=5)

Mg/dl – milligram per deciliter, SD – Standard deviation, n = number of animals per group

Our result in Table ii show that none of the methods used for animal sacrificed altered the kidney function enzymes compared to those of the control group. Urea and Creatinine levels however actually increased in groups B, C and D compared to the control group A, although the increases were not statistically significant. It is therefore possible that with longer exposure to these chemicals, there may be further increases, leading to statistically significant results.

The present study has been carried out using male Wistar rats. One of our major challenges in sub-Saharan Africa in conducting research is in methodology owing to deficiency of adequate equipment and standard laboratories to conduct researches. Therefore, on most occasions we make do with what is readily available. This however, may impact adversely on the final outcome of our researches. We therefore seek to create this awareness as regards organ of study and method of sacrifice.

Our results presented in tables i and ii shows that cervical dislocation did not negatively affect Liver and Kidney function enzymes as well as the cytoarchitecture of these organs in Wistar rats. This is supported by the works of Carbone et al. (2012) which showed that cervical dislocation was a considerably humane method in mice sacrifice, although the procedure may fail to achieve euthanasia sometimes. Also, Roustan et al. (2012) reported that cervical dislocation is the best method of mouse euthanasia for obtaining intact oocytes for biomedical research. Kato et al. (2013) however suggests that cervical dislocation should not be the method of choice when studying fetuses. However, this was not the case with diethyl ether and formalin inhalation. Our results show that serum levels of AST, ALT, ALP as well as urea (Ur) and creatinine (Cr) appeared unaffected and were comparable to those of the control for rats in groups C and D. This implies that the use of diethyl ether and formalin inhalation as methods of rat sacrifice did not adversely affect the liver function and kidney function enzymes. This is supported by the works of Goodies et al. (2015). However, the works of Brojeni et al. (2018) showed that exposure o diethyl ether may affect paraventricular nuclei glycosensing neurons-induced food intake in fasted rats. We can therefore say based on these results only that diethyl ether is safe for use as an agent of euthanasia when studying the liver and kidney.

**Result of Histological Studies (H&E)**

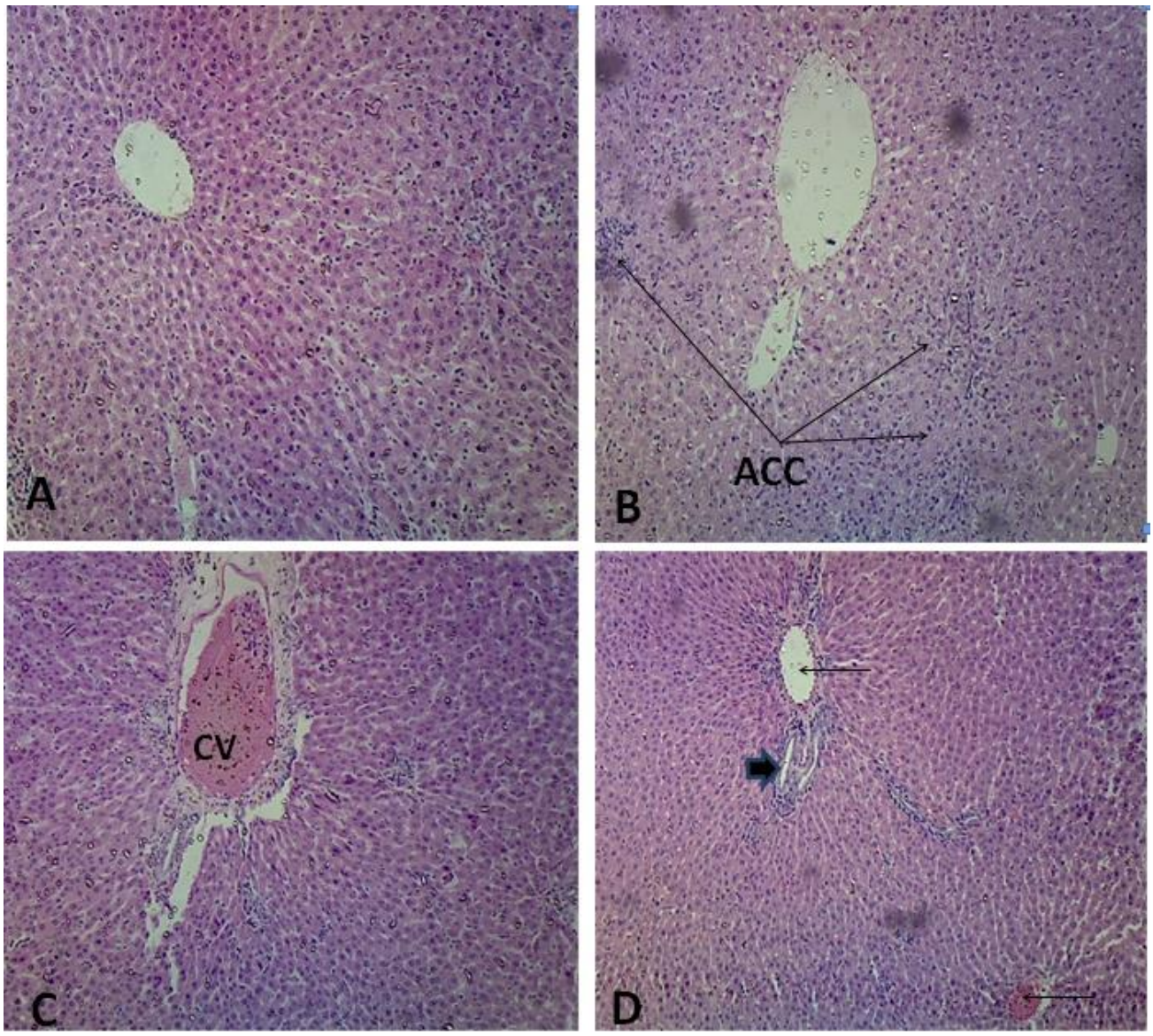


**Plate 1**: Photomicrograph of rat liver. A represent rats in the control group. The liver tissue appears normal with the central vein (CV) and radiating hepatocytes and sinusoidal spaces. B represents rat liver from group B showing several areas of cellular congestion (ACC) and inconspicuous porta triad. Slide C shows cellular hypertrophy and cellular congestion around the central vein. It also shows distorted porta triad (arrow head). D on the other hand showed cellular shrinkage with shrunken central vein (arrows) and porta triad (arrow head).

The result of our histological studies lends some support to the results of the serum chemistry presented in table i. Chloroform sedation used in the sacrifice of group B animals presents adverse effects on the cytoarchitecture of rat liver. Cellular distortion is also appreciable in the other groups C and D exposed to diethyl ether and formalin except for the control group sacrificed by cervical dislocation. Although the serum chemistry result showed no significant adverse effect on the liver function enzymes, it was beginning to follow a trend that might have materialized to functional distortion had we exposed them for longer time.

Plate 2 also shows that apart from cervical dislocation used in group A, the other groups had one form of tissue distortion or the other, ranging from cellular and parenchymal shrinkage to infiltrations and glomerular disorientation. There are therefore indications that chloroform inhalation, diethyl ether inhalation and formalin inhalation used as methods of sacrifice may alter tissue cytoarchitecture of liver and kidney, thereby leading to misinterpretation and false positive results in histological and histopathological reports. This is however the practice with 6.25% for diethyl ether and 15% for formalin. In all, about a total of

21.25% of clinical anatomists in Nigeria might be making wrong inferences when reporting on the kidney or liver, based on erroneous method of sacrifice.

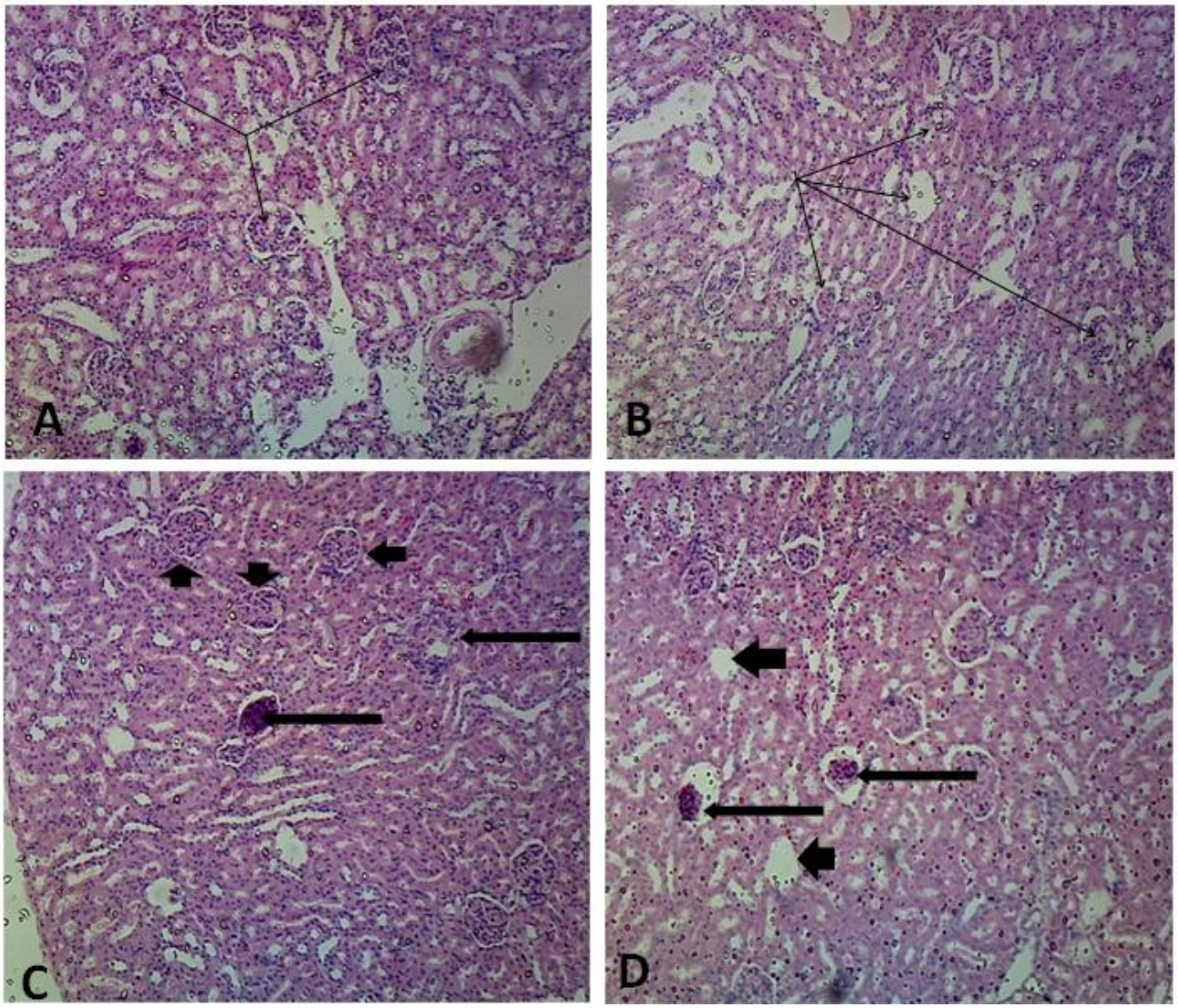


**Plate 2**: Plate 1: Photomicrograph of rat kidney. A- represents kidney tissue from the control group showing normal glomeruli (arrows) with normal cortical architecture. B - represents kidneys of rats in group B showing several distorted glomeruli and signs of tissue infiltration. C represents kidneys from rats in group. C - showing areas of cellular congestion (arrows), Blurry glomeruli (arrow) as well as narrowing of the urinary spaces (arrow heads) atypical of a normal kidney tissue. D - show photomicrographs of rat kidney with some empty renal corpuscles (arrow heads) and distorted glomeruli with wide urinary spaces (arrows).

The present study has been carried out using rats. Our preliminary results indicated that 90% of clinical anatomists use rats for their research works. The other 2.5% and &.5% use mice and rabbits respectively. One of our major challenges in sub-Saharan Africa in conducting research is in methodology owing to deficiency of adequate equipment and standard laboratories to conduct researches. Therefore, on many occasions we make do with what is readily available. This however may impact negatively on the final outcome of our researches. We therefore seek to evaluate procedural accuracy and authenticity as regards method of animal sacrifice and its suitability for particular organ of study (liver and kidney).

Our result presented in table i and ii above shows that cervical dislocation, diethyl ether and formalin inhalation did not negatively affect liver and kidney function enzymes. However, chloroform inhalation only significantly increased serum levels of alanine aminotransferase (ALT). This implies that exposure to chloroform fumes for as short as 3 to 4 minutes can adversely interfere with the results of liver enzymes. This is however the practice with 50% of our scientists. This implies that about 50% of clinical anatomists in Nigeria might be making wrong inferences when reporting on the liver if they used chloroform inhalation as the method of sacrifice.

Also, the result of our histological and histopathological studies shows cytoarchitectural distortion of the liver and kidney tissues following sacrifice by chloroform, formalin and diethyl ether inhalation. This increases the possibility of wrong inferences to 71.25%. This is therefore a serious research issue that needed to be addressed at all relevant quarters to enhance quality and outcomes of our researches from the largest black nation in the world.

We therefore have no doubt to report that although cervical dislocation appear safe as a method of rat sacrifice when studying the liver and kidney, chloroform, diethyl ether and formalin inhalation showed adverse effects on the histological features of the liver and kidney.

We therefore recommend that the other methods of anesthesia and euthanasia for rodent (especially rat) sacrifice be explored while the current methods be discouraged or applied with caution so as to improve on the quality of our research outcomes.

## 4. CONCLUSION

It can be concluded, based on the outcome of this research that cervical dislocation was safe for the liver and kidney as a method of sacrifice, both in chemistry and histopathology. However, chloroform sedation, diethyl ether sedation and formalin inhalation although appeared safe for serum chemistry were not so safe for histological studies.

**Recommendation**

It is therefore our recommendation that cervical dislocation is the best method out of the four common methods of sacrifice used by Nigerian researchers for serum chemistry as well as histology and histopathological studies of liver and kidney tissues.

**Conflict of Interests**

The authors declare that they have no conflicts of interest.

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